



dHAND-Cre transgenic mice reveal specific potential functions of dHAND during craniofacial development

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Abstract

Most of the bone, cartilage, and connective tissue of the craniofacial region arise from cephalic neural crest cells. Presumably, patterning differences in crest cells are a result of regional action of transcription factors within the developing pharyngeal arches. The basic helix–loop–helix transcription factor dHAND/HAND2 is expressed throughout much of the neural crest-derived mesenchyme of the pharyngeal arches, suggesting that it plays a crucial role in craniofacial development. However, targeted inactivation of the *dHAND* gene results in embryonic lethality by E10.5 due to vascular defects, preventing further analysis of the role of dHAND in cephalic neural crest cell development. In order to examine putative roles of dHAND during later stages of embryogenesis, we have used a transgenic lineage marker approach, in which a portion of the dHAND upstream region containing an enhancer that directs dHAND expression to the pharyngeal arches is used to drive *Cre* recombinase expression. By crossing these *dHAND*-*Cre* transgenic mice with *R26R* mice, we can follow the fate of cells that expressed *dHAND* at any time during development by examining β -galactosidase activity. We show that *dHAND* is first expressed in postmigratory cephalic neural crest cells within the pharyngeal arches. In older embryos, β -galactosidase-labeled cells are observed in most of the neural crest-derived lower jaw skeleton and surrounding connective tissues. However, labeled cells only contribute to substructures within the middle ear, indicating that our transgene is not globally expressed in cephalic neural crest cells within the pharyngeal arches. Moreover, *dHAND*-*Cre* mice will provide a valuable tool for tissue-specific inactivation of gene expression in multiple tissue types of neural crest origin.

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Introduction

Among events in skeletogenesis, craniofacial morphogenesis is perhaps the most intriguing, partially due to the origin of the bone, cartilage, and connective tissue that composes the face and neck. While bone and cartilage

throughout the body and skull vault are of mesodermal origin (Noden, 1983; Jiang et al., 2002), those in the facial region are derived from cephalic neural crest cells (Bronner-Fraser, 1995; Le Douarin, 1982; Noden, 1988). These cells arise along the lateral edge of the neural folds and begin a ventral migration around the time of neural tube closure, undergoing an epithelial-to-mesenchymal transformation during the process (Le Douarin et al., 1993; Serbedzija et al., 1992). They eventually come to reside in the pharyngeal arches, transient structures on the ventral aspect of the embryo, in which crest-derived ectomesenchymal cells con-

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tinue to proliferate and differentiate, eventually giving rise to adult craniofacial structures.

Numerous signaling molecules direct the patterning and development of cephalic neural crest cells and their derivatives (Francis-West et al., 1998; Mina, 2001; Wilkie and Morriss-Kay, 2001). These signals include Wnt1 and Wnt3a [expansion of premigratory neural crest cells prior to migration (Ikeya et al., 1997)], AP-2 [migration of crest cells to the arches (Schorle et al., 1996; Zhang et al., 1996)], and members of the *Distal-less* gene family [postmigratory patterning and differentiation (for reviews, see Kraus and Lufkin, 1999; Merlo et al., 2000)]. It is during this last postmigratory time period that soluble factors emanating from the overlying epithelium trigger signaling cascades within crest-derived ectomesenchymal cells that subsequently provide patterning and differentiation information necessary for development of bone and cartilage structures of the face and neck (Clouthier et al., 2000; Thomas and Sharpe, 1998; Tucker et al., 1999). These epithelial expression domains appear to be tightly controlled, thus providing much of the spatial specificity in transcription factor expression in the underlying mesenchyme. However, it is also believed that crest cells of specific lineages may also be programmed to express or respond to specific signals (Dorsky et al., 1998).

Two factors expressed in the pharyngeal mesenchyme are the basic helix–loop–helix transcription factors dHAND/HAND2 and eHAND/HAND1 (Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995). The genes encoding these factors are expressed in numerous locations within the embryo, including postmigratory crest cells in the arches. Their expression domains partially overlap, with *dHAND* expression observed throughout the mesenchyme of mandibular arch 1 and arches 2–6, whereas *eHAND* expression is confined to the ventral one-third of the mandibular arch one and arch two. While their expression patterns suggest that they might play crucial roles in craniofacial morphogenesis, targeted inactivation of the *dHAND* (Srivastava et al., 1997; Thomas et al., 1998) and *eHAND* (Firulli et al., 1998; Riley et al., 1998) genes in mice results in early embryonic lethality, thus preventing further analysis of their roles in cephalic neural crest cell development. However, some inferences on the role of *dHAND* can be made from studies in endothelin-A (ET_A) receptor-deficient embryos, in which *dHAND* expression within the arch mesenchyme is almost completely down-regulated (Clouthier et al., 2000). $ET_A^{-/-}$ embryos are born with severe craniofacial defects, including absence or malformation of the mandible, Meckel's cartilage, malleus, incus, and tympanic ring (Clouthier et al., 1998). While expression of at least five genes, including *dHAND* and *eHAND*, is disrupted in the arch mesenchyme of $ET_A^{-/-}$ embryos (Clouthier et al., 2000; and D.E.C., unpublished data), the *dHAND* expression domain encompasses most of the expression domains of other affected genes, suggesting

that dHAND may play a dominant role in development of craniofacial bones and cartilages.

Recently, we have shown using an 11-kb fragment of the *dHAND* upstream region driving transgenic expression of *lacZ* that *dHAND* expression is observed in facial bones and cartilage until at least E16.75 (Charité et al., 2001). These included portions of the mandible and Meckel's cartilage, as well as the tongue musculature and connective tissue of the lower jaw. To further examine potential roles of dHAND during craniofacial development, we have undertaken a lineage analysis of cells that express *dHAND* at any time during development. To accomplish this, we created transgenic mice in which a portion of the dHAND upstream region containing elements necessary for expression in the pharyngeal arch mesenchyme was used to drive *Cre* recombinase expression. By crossing *dHAND-Cre* transgenic mice with mice from the Cre reporter line *R26R* (Soriano, 1999), we determined the fate of dHAND-lineage cells by staining for β -galactosidase activity and then compared the staining with both staining in *dHAND-lacZ* transgenic embryos and endogenous *dHAND* expression as judged by in situ hybridization. We find that, during craniofacial morphogenesis, *Cre* expression in *dHAND-Cre;R26R* embryos is first detected in neural crest-derived pharyngeal arch mesenchyme at E9.5, matching both the expression of the *dHAND-lacZ* transgene and endogenous *dHAND* expression. In older *dHAND-Cre;R26R* embryos, labeled cells become restricted to a limited number of neural crest-derived structures, including Meckel's cartilage, mandibular bone, dental pulp of mandibular teeth, and the mesenchyme surrounding the developing submandibular gland buds. Most striking is the restriction of staining observed in the middle ear ossicles, with only the manubrium of the malleus containing labeled cells. No cells are observed in the body of the malleus, incus, or stapes. In *dHAND-lacZ* embryos, a similar distribution of labeled cells is observed in most structures, though apparent differences in fated vs active expression exist within craniofacial cartilages and mandibular teeth. We observe very little endogenous *dHAND* expression in older embryos, with the primary exception being around the mandibular incisors. Together, our findings illustrate both the specificity and complexity of *dHAND* expression and function.

Materials and methods

Transgenic constructs

The plasmid pOG231 contains a CMV promoter, a synthetic intron, the Cre cDNA, a nuclear localization signal (NLS), and a SV40 polyadenylation signal (O'Gorman et al., 1997). To create the *dHAND-Cre* transgene, pOG231 was digested with *Xho*I, treated with Klenow enzyme to produce blunt ends, and then digested with *Eco*RI. This scheme liberated a 1.8-kb fragment containing the synthetic

intron, Cre-NLS, and polyadenylation sequences. This fragment was then subcloned into pSP73 (Promega) cut with *EcoRI* and *EcoRV*, resulting in the plasmid pSP73-Cre-NLS. This plasmid was then digested with *KpnI*, treated with T4 DNA polymerase to produce blunt ends, followed by digestion with *XhoI* and gel purification. To prepare the promoter insert, pBSdHAND-lacZ (Charité et al., 2001), containing 11 kb of the dHAND upstream region, was cut with *SfiI* (3' to the dHAND transcription initiation site), treated with T4 DNA polymerase to produce blunt ends, followed by restriction with *XhoI* (7.4 kb upstream of the *SfiI* site). The isolated 7.4-kb *SfiI* (b)–*XhoI* fragment was then subcloned into pSP73-Cre-NLS [*KpnI* (b)–*XhoI*]. The resulting plasmid, pdHAND-Cre, was verified by restriction digest and partial sequencing. The final 9.2-kb construct was isolated by restriction enzyme digest with *SalI*. The fragment was isolated on a 0.8% ultra-pure agarose gel (FMC), purified by perchlorate elution, and quantified (Comerford et al., 1995).

Production of transgenic mice

Construction and characterization of the –11-kb *dHAND-lacZ* transgenic mice has previously been described (Charité et al., 2001; McFadden et al., 2000). To construct *dHAND-Cre* transgenic mice, the purified dHAND-Cre construct was injected at 3 ng/μl into C57BL6/J × SJL F₂ fertilized one-cell eggs, and two-cell embryos were transferred to pseudopregnant females as previously described (Clouthier et al., 1997). Initial genotyping of putative founders was performed by dot blot hybridization. Five founder mice were identified, with transgene copy number ranging from 1 to 10 copies per cell. Founder mice were bred to mice from the Cre reporter mouse strain *R26R* (strain 129/Sv-*Gtosa26^{tm/Sor}*; The Jackson Laboratory, Bar Harbor, ME) (Soriano, 1999). Embryos were collected at E10.5 and analyzed for β-galactosidase (β-gal) staining to evaluate the fidelity and robustness of *Cre* expression. Three lines were established that produced compound hemizygous pups with β-gal activity. While all three lines showed similar expression patterns, embryos from lines 7-1 and 7-2 had the most robust staining. Therefore, these lines were used for the current study.

Genotyping of dHAND-Cre transgenic mice and embryos

Genotyping was performed by PCR using genomic DNA isolated from a small tail biopsy or embryonic yolk sac. The presence of the *dHAND-Cre* transgene was determined by using the Cre primers 5'-GGACATGTTTCAGGGATCGC-CAGGCG-3' and 5'-GCATAACCAGTGAAACAGCATT-GCTG-3' as previously described (Kisanuki et al., 2001). The presence of the *R26R* allele was determined by using primers 5'-GCGAAGAGTTTGTCTCAACC-3' (R1295) and 5'-AAAGTCGCTCTGAGTTGTTAT-3' (R26RF) as previously described (Soriano, 1999). The presence of the

dHAND-lacZ transgene was determined by using the lacZ primers 5'-GACACCAGACCAACTGGTAATGG-3' and 5'-GCATCGAGCTGGGTAATAAGCG-3'. Bands were visualized on 1% agarose gels.

β-Galactosidase staining

For whole-mount staining [embryonic day (E) 8.5 to E11.5], embryos were collected and fixed for 1 h in 4% paraformaldehyde on ice. Embryos were then rinsed in lacZ rinse buffer (0.2 M sodium phosphate, pH 7.3, 2 mM magnesium chloride, 0.02% NP-40, 0.01% sodium deoxycholate) twice for 10 min and then placed in lacZ staining buffer (lacZ rinse buffer plus 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal) and incubated overnight in the dark at room temperature. Embryos were rinsed in PBS, postfixed in 10% neutral-buffered formalin for 4 h, rinsed in PBS, and stored in 70% ethanol. Embryos were analyzed and photographed on an Olympus SZX12 stereomicroscope fitted with a DP11 digital camera. To observe β-gal staining at the cellular level, some of these stained embryos were dehydrated through graded ethanols and Cedarwood oil (Polysciences) and then embedded in paraffin and sectioned at 12 microns on a Lecia RM2135 microtome. Sections were collected on positively charged Superfrost Plus microscope slides (Fisher Scientific), partially rehydrated, counterstained with nuclear fast red, dehydrated, and coverslipped with DPX mounting media (BDH). Analysis of sections was performed by using a Nikon E600 microscope with an attached SPOT RT digital camera.

For staining of older embryos (E13.5–E18.5), embryos were isolated into PBS and immediately snap frozen in OCT on a dry ice/95% ethanol bath. Embryos were sectioned at 14–18 microns on a CM1900 cryostat (Leica). Sections were collected on Superfrost Plus slides, air dried for 2 h, fixed in 0.5% glutaraldehyde at room temperature for 30 min, washed three times for 20 min each in lacZ rinse buffer, and incubated overnight in lacZ staining solution at 37°C. After rinsing in PBS, slides were counterstained in nuclear fast red (E8.5–E15.5) or eosin (E18.5) and coverslipped with PBX mounting media. Analysis and photography was performed as described above.

In situ hybridization analysis

Sectional in situ hybridization analysis was performed as previously described (Shelton et al., 2000) by using a ³⁵S-UTP-labeled riboprobe against dHAND (Srivastava et al., 1997), with the hybridization carried out at 70°C in hybridization buffer containing 0.75 M NaCl. Sections were exposed for 2–3 weeks before developing, fixing, and counterstaining with Richard Allen hematoxylin 2 (VWR). Whole-mount in situ hybridization analysis was performed as previously described by using a digoxigenin-labeled riboprobe against dHAND (Clouthier et al., 2000). Following

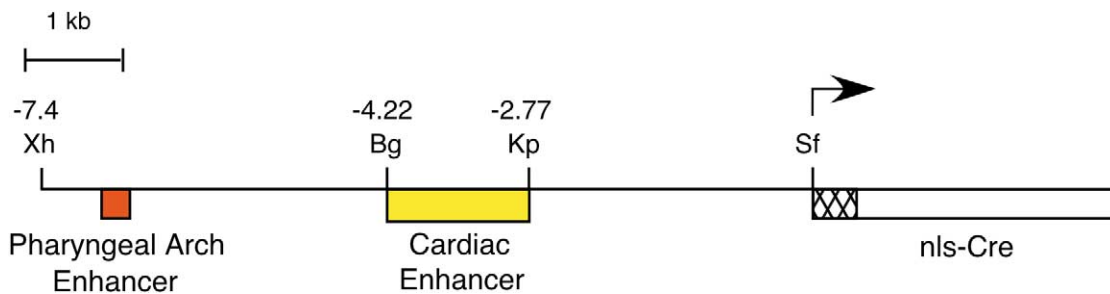


Fig. 1. Diagram of the *dHAND-Cre* transgene. A 7.4-kb fragment of the *dHAND* upstream region, containing enhancers required for pharyngeal arch and cardiac expression, was fused to a *Cre* cDNA containing nuclear localization and SV40 polyadenylation sequences. A synthetic intron resides in the 5' end of the *Cre* cDNA to aid transgene expression. Bg, *Bgl*II; Kp, *Kpn*I; Sf, *Sfi*I; Xh, *Xho*I.

staining for alkaline phosphatase activity, embryos were photographed, embedded in paraffin, and sectioned at 16 microns. Sections were not counterstained. Photography was performed by using Nomarski DIC optics.

Results

Creation of *dHAND-Cre* transgenic mice

We initially set out to create a mouse strain in which we could follow the fate of *dHAND*-expressing cells throughout embryonic development. To accomplish this, we constructed a transgene in which a 7.4-kb fragment of the *dHAND* upstream region, which contains enhancer elements necessary for both cardiac and pharyngeal arch expression of *dHAND* (Charité et al., 2001; McFadden et al., 2000), was used to drive expression of Cre recombinase (Fig. 1). Microinjection of this construct resulted in five transgenic founder mice. To assess expression of the transgene, founder animals were bred with mice from the *R26R* Cre reporter line (Soriano, 1999). Expression of *Cre* in *R26R* mice results in activation of a *lacZ* gene and thus robust β -galactosidase (β -gal) staining, providing an indelible marker of cells that have expressed *dHAND* at any time during development. E10.5 *dHAND-Cre;R26R* embryos were collected from each line and assayed by β -gal staining. Three lines showed staining in the heart and pharyngeal arches in a *dHAND*-specific manner (data not shown); two of these (7-1 and 7-2) were subsequently used in the analyses described below. Findings were identical in both lines, though overall intensity of expression was less in the 7-2 line.

dHAND-Cre expression during early embryogenesis

We initially examined β -gal staining in early *dHAND-Cre;R26R* embryos. Since β -gal staining could represent either active or past *dHAND* enhancer activity, we also examined β -gal staining in -11.0 -kb *dHAND-lacZ* transgenic mouse embryos and compared that with the endogenous *dHAND* expression pattern. Though our *dHAND-lacZ*

transgenic mouse line contained an additional 3.6 kb of upstream promoter region, both *dHAND-lacZ* and *dHAND-Cre* transgenic strains contained the same pharyngeal arch enhancer. Further, in our previous studies, -11.0 kb *dHAND-lacZ* and -7.4 kb *dHAND-lacZ* embryos had identical β -gal staining patterns in the developing pharyngeal arches (Charité et al., 2001).

Staining was first observed in *dHAND-Cre;R26R* embryos at E7.75 in the cardiac crescent (data not shown), matching the endogenous *dHAND* expression pattern (Srivastava et al., 1997). By E8.5, expression was observed in the lateral plate mesoderm and common ventricle of the heart but not in the pharyngeal arches, a finding recapitulated in *dHAND-lacZ* embryos (data not shown) and matching the endogenous *dHAND* expression pattern (Charité et al., 2000; Srivastava et al., 1997). However, the staining in the lateral plate mesoderm of *dHAND-Cre;R26R* was more restricted than the endogenous *dHAND* expression pattern. By E9.5, labeled cells in *dHAND-Cre;R26R* embryos were observed within much of the neural crest-derived mesenchyme of mandibular arch 1, with scattered cells also observed in the medial mesenchyme of arch 2 (Fig. 2A). Labeled cells were not observed in the overlying arch epithelium (Fig. 2A'). This staining pattern was identical to that observed in *dHAND-lacZ* embryos, though labeled cells were primarily confined to arch one (Fig. 2B and B'). β -gal staining in both of these transgene lines closely matched the endogenous *dHAND* expression pattern (Fig. 2C and C'), suggesting that the arch staining in *dHAND-Cre;R26R* embryos represented active *dHAND* expression.

β -gal staining in E9.5 *dHAND-Cre;R26R* embryos was also observed in the neural crest-derived cervical dorsal root ganglia (DRG) (black arrow in Fig. 2A), a site that is not stained in *dHAND-lacZ* embryos (Fig. 2B) and that does not show any endogenous expression (Fig. 2C). These findings, along with the fact that the degree of labeling in the DRGs differed in different *dHAND-Cre;R26R* lines examined, suggest that the DRG staining is artifactual. Labeled cells in *dHAND-Cre;R26R* embryos were also present in the foregut and forelimb buds. Expression in the forelimb bud was unusually strong for the embryonic age of the embryo in comparison with the endogenous limb expression pattern of

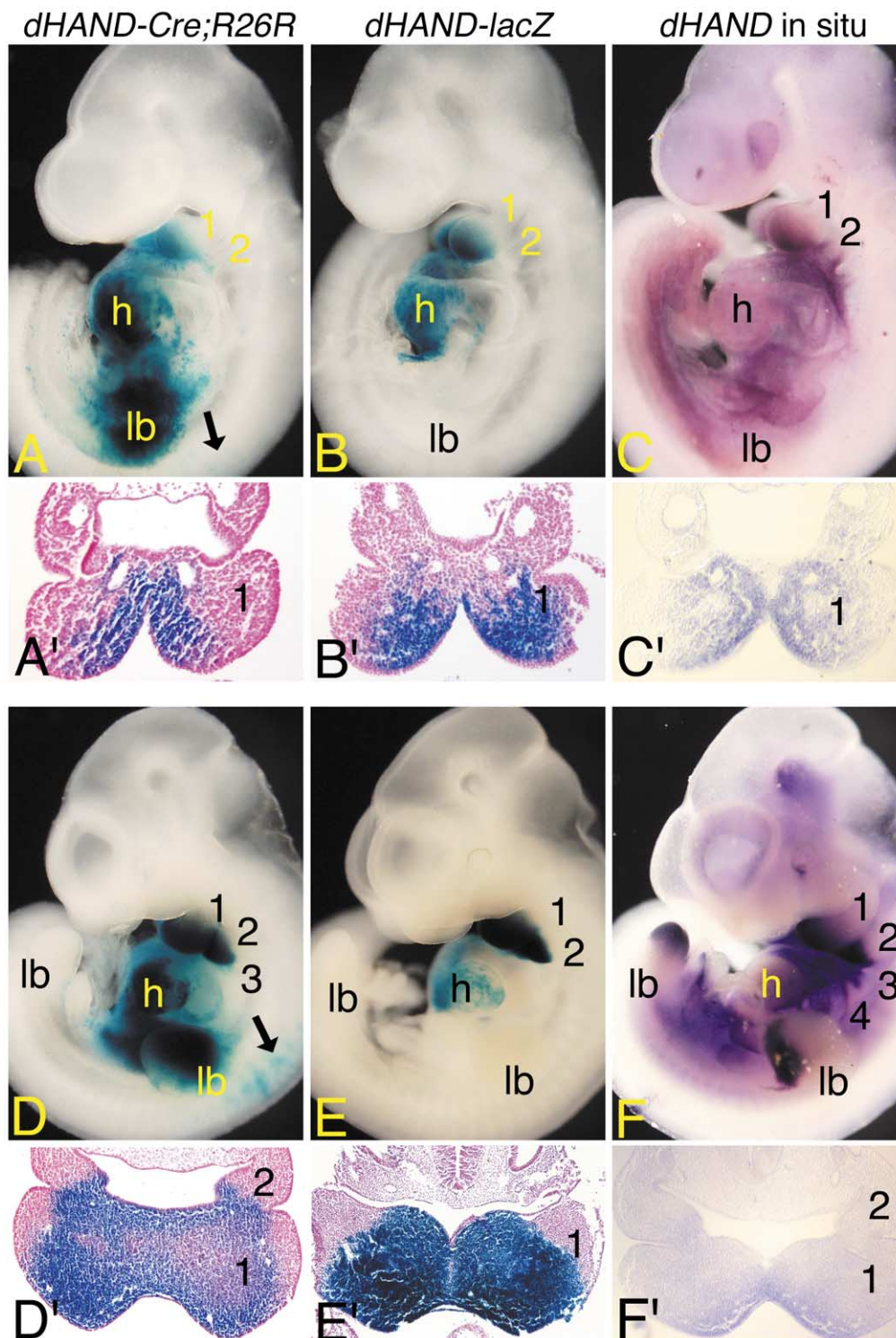


Fig. 2. Early *dHAND* expression domains in *dHAND-Cre; R26R* embryos. (A–F) Lateral (A–F) and transverse (A'–F') views of *dHAND-Cre;R26R* (A, A'; D, D') and *dHAND-lacZ* (B, B'; E, E') embryos stained in whole mount for β -gal activity and wild type embryos (C, C'; F, F') after whole-mount in situ hybridization analysis of *dHAND* expression. Transverse sections of β -gal-stained embryos were counterstained with nuclear fast red and photographed by using brightfield optics. Transverse sections of whole-mount in situ embryos were photographed by using Nomarski DIC optics. (A) In E9.5 *dHAND-Cre; R26R* embryos, labeled cells are observed in the first mandibular (1) and second (2) arches, common heart ventricle (h), and forelimb bud (lb). Few scattered cells are observed in the early dorsal root ganglion (black arrow). (A') A transverse section through the first arch of the embryo shown in (A) illustrates that stained cells are confined to the arch mesenchyme in a *dHAND*-specific pattern. (B) In E9.5 *dHAND-lacZ* embryos, staining is observed in the mandibular arch and common heart ventricle. (B') A transverse section through the first arch of the embryo shown in (B) shows that labeled cells are confined to the arch mesenchyme. (C) In E9.5 wild type embryos, *dHAND* expression is observed in the first mandibular and second arches and in the future right ventricle of the heart (obscured by the left ventricle). (C') As observed in (A') and (B'), *dHAND* expression is confined to the arch mesenchyme. (D) By E10.5, labeled

dHAND (Fig. 2C; and Charité et al., 2000; Fernandez-Teran et al., 2000; Srivastava et al., 1995). Further, while the endogenous *dHAND* gene is expressed in the posterior limb bud, labeled cells in E9.5 *dHAND-Cre;R26R* embryos were observed in the anterior portion of the forelimb. There was no apparent labeling of limb bud mesenchyme in *dHAND-lacZ* embryos (Fig. 2B).

By E10.5, the more caudal arches in *dHAND-Cre;R26R* embryos also contained labeled cells; other sites of expression remained unchanged (Fig. 2D). Within the arches, labeled cells appeared denser around the arch periphery (Fig. 2D'), resembling the pattern previously observed for neural crest cells (Chai et al., 2000). With the exceptions noted at E9.5, the overall expression pattern still resembled β -gal staining in *dHAND-lacZ* embryos (Fig. 2E and E') and endogenous *dHAND* expression (Fig. 2F and F'), though very few labeled cells were observed in arches 3–6 of *dHAND-lacZ* embryos. These findings suggest that the arch staining in *dHAND-Cre;R26R* embryos was still a result of active *dHAND* expression. While staining in locations other than the arches or derivatives continued throughout development in *dHAND-Cre;R26R* embryos, the majority of the present study focuses on findings during craniofacial development.

Fate of cells from a dHAND lineage during orofacial development

Using *dHAND-lacZ* transgenic mice, we have previously shown that *dHAND* is expressed in subsets of cells during orofacial development (Charité et al., 2001). To more directly determine the participation of *dHAND* lineage cells in this process, we examined β -gal staining in E13.5 and E18.5 *dHAND-Cre;R26R* embryos and compared that to both β -gal staining in *dHAND-lacZ* embryos and endogenous *dHAND* expression in wild type embryos. In E13.5 *dHAND-Cre;R26R* and *dHAND-lacZ* embryos, Meckel's cartilage and surrounding perichondrium, as well as mandibular bone and surrounding connective tissue, all neural crest derivatives, were composed of labeled cells (Fig. 3A and B). The mesenchyme surrounding the buds and ducts of the developing submandibular gland (pseudoglandular stage), a neural crest derivative (Jaskoll et al., 2002), was also composed of β -gal-stained cells (see insets, Fig. 3A' and B'). The ductal epithelium did not contain labeled cells. Mesodermal derivatives also contained labeled cells, in-

cluding the lamina propria of the tongue. One notable difference was that Meckel's cartilage in *dHAND-lacZ* embryos was composed of both labeled and unlabeled cells, compared with almost complete contribution of labeled cells in *dHAND-Cre;R26R* embryos. Endogenous *dHAND* gene expression was observed in the lamina propria of the tongue and in the submandibular gland mesenchyme, though the latter appeared to be more restricted than observed in *dHAND-lacZ* embryos (Fig. 3C). Expression in the early mandibular bone and Meckel's cartilage was virtually undetectable. The other major site of labeled cells in E13.5 *dHAND-Cre;R26R* embryos was in the developing scapula, in which labeled cells composed the entire coracoid process and acromion of the scapula, but were mixed with unlabeled cells in the scapula blade (data not shown). The significance of this scapular staining is unclear, as endogenous *dHAND* expression was not observed in these structures at E13.5 (data not shown).

By E18.5, the mandibular bone and surrounding lamina propria of *dHAND-Cre;R26R* embryos was composed of labeled cells along its entire proximodistal axis (Fig. 3D and G; and data not shown). Likewise, Meckel's cartilage was also stained along its entire axis (Fig. 3D and G; and data not shown), as were the chondrocytes and surrounding perichondrium composing the anterior and lateral processes of the proximal mandible (Fig. 3G). The extent of cell labeling decreased near the junction of the processes with the mandibular bone, a zone composed of hypertrophic and presumably ossified chondrocytes (see inset, Fig. 3G'). Labeled cells were also observed throughout the tongue, with strongest staining again in the lamina propria (Fig. 3D), though the relative number of labeled cells appeared greater in the oral portion (distal two-thirds) than in the pharyngeal portion (proximal one-third) of the tongue (data not shown).

In *dHAND-lacZ* embryos, labeled cells were also observed throughout the proximodistal axis, though the relative presence of cells was higher in the caudal portion of the mandible along this axis (Fig. 3E). Labeled cells were also present in Meckel's cartilage, though they again were mixed with unlabeled cells (Fig. 3E and H). Very few labeled cells were observed in the mandibular processes, though strong staining was observed in the perichondrium around both these processes and Meckel's cartilage (Fig. 3E and H). Endogenous *dHAND* expression was only observed in scattered cells within the mandible (Fig. 3F and I). Expression

cells in *dHAND-Cre;R26R* embryos are observed in arches 1–3, the ventricle, and the limb bud. Scattered cells are also observed in the atrium. While not visible in this view, stained cells are also observed in arch 4. Labeled cells are also observed in the cervical dorsal root ganglion (black arrows). (D') In a transverse section through the first and second arches, staining is observed in the arch mesenchyme, with the intensity of staining highest in the periphery of the arch. (E) In E10.5 *dHAND-lacZ* embryos, labeled cells are observed in arches one and two and in the ventricles of the heart. Scattered cells are also present in the atria. (E') As observed in *dHAND-Cre;R26R* embryos, staining within the mesenchyme of the first arch is strongest at the periphery. While not shown due to a slight difference in the angle of section, the second arch staining in *dHAND-lacZ* embryos is comparable with that of *dHAND-Cre;R26R* embryos. (F) *dHAND* expression in E10.5 wild type embryos is observed in arches 1–4, distal limb buds, and right ventricle of the heart. (F') A section through the first and second arch again illustrates staining throughout the medial half of the arch mesenchyme, with the strongest staining observed along the periphery of the arch.

was not observed in the perichondrium of the mandibular processes.

Contribution of dHAND lineage cells in middle ear and throat structures

Much of the external and middle ear is composed of ectomesenchymal cells derived from the first and second arches (Fekete, 1999; Kontges and Lumsden, 1996; Mallo, 2001; Noden, 1988). In E13.5 *dHAND-Cre;R26R* embryos, most of the mesenchyme composing the connective tissue in the external region was composed of β -gal-positive cells (Fig. 4A), though the epithelium of the future external auditory meatus (derived from the first pharyngeal cleft) was unstained (arrow). In the middle ear, labeled cells were observed in Meckel's cartilage as described above, though a strict delineation of labeled and unlabeled cells occurred at the junction with the cartilaginous precursor of the malleus, which was unlabeled (data not shown). However, labeled cells were observed in the distal malleus (Fig. 4A). Further, while most of the stapes was composed of unlabeled cells, the caudal stapes and surrounding perichondrium did contain a mixture of labeled and unlabeled cells (data not shown). We also observed labeled cells in the sympathetic ganglion, though there was extensive mixing with unlabeled cells (see inset, Fig. 4A'). Other sites of expression included the cervical dorsal root ganglia, superior cervical sympathetic trunk, and ventral primary ramus of the cervical nerve (data not shown). As observed for the sympathetic ganglion, contribution of labeled cells within these structures was scattered.

A similar pattern of β -gal staining was observed in *dHAND-lacZ* embryos, though labeled cells in Meckel's cartilage were mixed with unlabeled cells (Fig. 4B), while the sympathetic ganglion was composed solely of unlabeled cells (see inset, Fig. 4B'). This pattern of labeled cells was not necessarily reflected in the endogenous expression of *dHAND*, as very little expression was observed in the external or middle ear, though intense signal was present in the sympathetic ganglion and the mandibular branch of the trigeminal nerve (*) (Fig. 4C).

At E18.5, the tympanic ring and gonial bones, neural crest-derived structures formed by intramembranous ossification, were composed of labeled cells in *dHAND-Cre;R26R* embryos, as was the surrounding connective tissue and Meckel's cartilage (Fig. 4D). Of the middle ear ossicles, only the manubrium of the malleus contained labeled cells; the cartilaginous precursors of the incus or stapes were unlabeled (Fig. 4G and J). Other elements composed of labeled cells included the distal portion of the styloid cartilage and cartilage associated with the pinna, with scattered cells also present in the sympathetic ganglion (Fig. 4M and 4M'). Labeled cells in E18.5 *dHAND-lacZ* embryos were observed in a similar pattern, with staining observed in the tympanic and gonial bones (Fig. 4E), Meckel's cartilage (Fig. 4E), the manubrium of the malleus (Fig. 4H), and the

styloid cartilage (Fig. 4N). However, as observed at E13.5, labeled cells in cartilage structures, while composing most of the perichondrium around the structures, were extensively mixed with unlabeled cells within the cartilage. This was most prominent in the styloid cartilage (Fig. 4N). Labeled cells were not present in the sympathetic ganglion (Fig. 4N). Endogenous *dHAND* expression was not widely observed, with virtually no expression observed in any of the middle ear structures examined (Fig. 4F, I, and L). However, as observed at E13.5, expression was observed in the sympathetic ganglion (Fig. 4O).

Labeled cells were also observed in the second and third arch derivatives of *dHAND-Cre;R26R* embryos. The hyoid bone of the throat is actually derived from three cartilaginous precursors derived from both arches. The lesser horns, a second arch derivative, were composed exclusively of labeled cells (Fig. 4P). However, the greater horns, a third arch derivative, were composed of both labeled and unlabeled cells (data not shown). The body of the hyoid, derived from both arches (Kontges and Lumsden, 1996), was also composed of a mixture of labeled and unlabeled cells in the cartilaginous portions (endochondral ossification begins in the middle of the body and spreads bilaterally). However, fewer hypertrophic chondrocytes and presumably ossified chondrocytes were labeled, similar to our observations in the mandibular processes. Scattered labeled cells were observed in the fourth arch-derived thyroid cartilage (data not shown). Labeled cells were also present in the mesenchyme composing the submandibular and sublingual glands (arrows in Fig. 4P) and in adipose tissue of the neck (see inset, Fig. 4P').

In E18.5 *dHAND-lacZ* embryos, β -gal cells were observed in the perichondrium surrounding the lesser horns of the hyoid, with few labeled cells actually composing the lesser horns or hyoid body (Fig. 4Q). Labeled cells were also present in the submandibular and sublingual gland mesenchyme (arrow in Fig. 4Q), but were absent in adipose tissue (see inset, Fig. 4Q'). In contrast to this staining pattern, very little *dHAND* expression was detected by in situ hybridization in any hyoid structures (Fig. 4R), in stark contrast to the strong signal detected in adipose tissue. Expression was also detected in the submandibular and sublingual glands (see inset, Fig. 4R').

Expression of dHAND during odontogenesis

During odontogenesis, cephalic neural crest cell-derived ectomesenchyme contributes to the formation of odontoblasts (and consequently dentin matrix), dental pulp, cementum, and the periodontal ligaments of the incisors and molars (Chai et al., 2000). To examine potential roles of *dHAND* signaling in this process, we examined the extent of β -gal-stained cells at different stages of tooth development in *dHAND-Cre;R26R* embryos. As with our other analyses, we compared this staining with that observed in age-matched *dHAND-lacZ* embryos and with endogenous

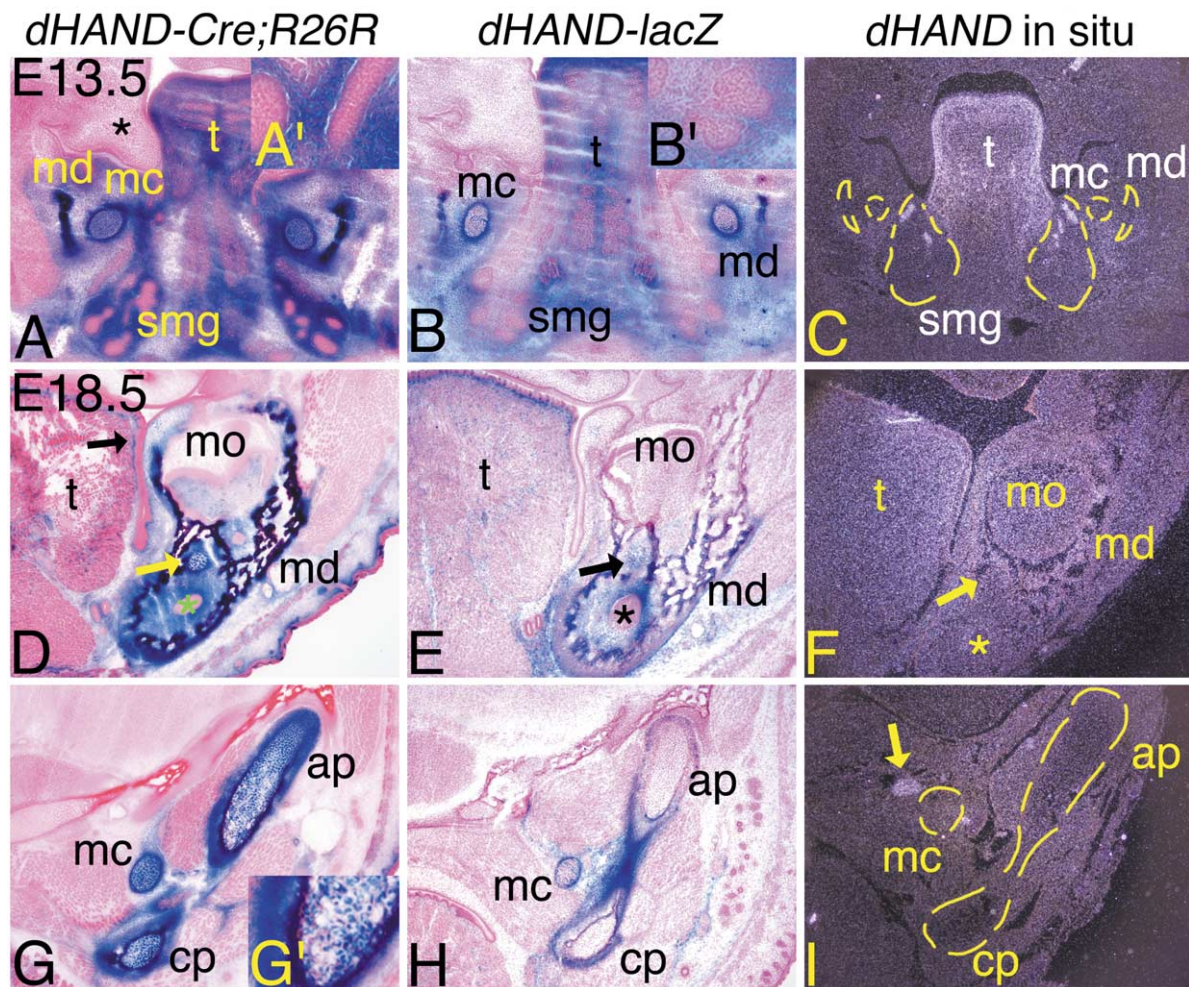
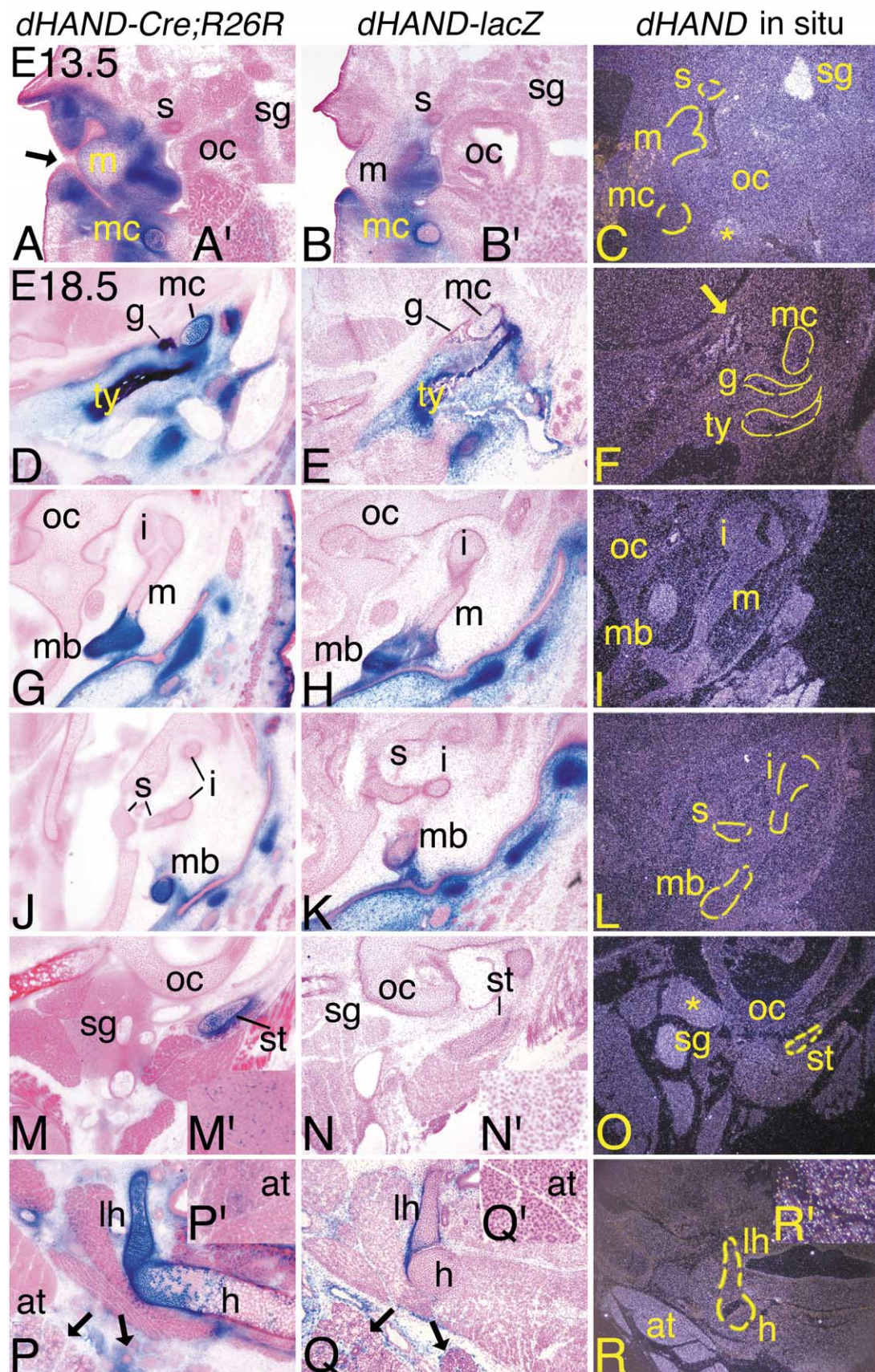


Fig. 3. Contribution of dHAND lineage cells to arch-derived structures of the jaw of *dHAND-Cre;R26R* embryos. Transverse (A–C) and frontal (D–I) sections through the heads of *dHAND-Cre;R26R* (A, D, G), *dHAND-lacZ* (B, E, H), and wild type (C, F, I) embryos. Frozen sections from *dHAND-Cre;R26R* and *dHAND-lacZ* embryos were stained for β -gal activity and counterstained with nuclear fast red (E13.5) or eosin (E18.5). Paraffin sections from wild type embryos were probed with a ^{35}S -labeled riboprobe against *dHAND*. After developing, sections were counterstained with Harris hematoxylin and photographed under darkfield conditions. To aid in the visualization of specific structures, yellow lines have been added to some of the darkfield panels. (A–C) Transverse sections through the lower jaw of E13.5 embryos. (A) Labeled cells in *dHAND-Cre;R26R* embryos are observed in structures derived from the first pharyngeal arch, including mandibular bone (md), Meckel's cartilage (mc), tongue (t), and surrounding mesenchyme/connective tissue. Abundant labeled cells are also observed in the mesenchyme of the developing submandibular glands (smg), but not in the glandular epithelium (see inset, A'). The mesenchyme of the upper jaw (future maxilla; *) and oral cavity epithelium do not contain labeled cells. (B) Labeled cells in *dHAND-lacZ* transgenic embryos are observed in a similar pattern to those in *dHAND-Cre;R26R* embryos, though labeled cells in Meckel's cartilage are mixed with unlabeled cells. (C) Endogenous *dHAND* expression is observed in the lamina propria of the tongue and in the mesenchyme surrounding the proximal ducts of the submandibular gland. Very little expression is observed in the mandible or Meckel's cartilage. (D–I) Frontal sections through the mandible of E18.5 embryos. (D) β -gal-stained cells in *dHAND-Cre;R26R* embryos are present in the connective tissue of the tongue (t), with highest staining in the lamina propria (black arrow). Almost all cells in the mandible, Meckel's cartilage (yellow arrow), and surrounding mesenchyme are also labeled. (E) Labeled cells in *dHAND-lacZ* embryos are again observed in a similar pattern to those in *dHAND-Cre;R26R* embryos. However, a mixture of labeled and unlabeled cells composes the mandibular bone and Meckel's cartilage. (F) Faint *dHAND* expression is observed in the mandibular bone. Very little expression is observed in Meckel's cartilage. The asterisks in (D–F) mark the enamel organ of the left incisor. (G) In a more proximal section to that shown in (D–F), labeled cells in *dHAND-Cre;R26R* embryos are observed in Meckel's cartilage and in the articular (ap) and coronoid (cp) processes of the mandible and surrounding perichondrium. However, fewer labeled cells are observed near the junction with the ossified mandible, an area undergoing endochondral ossification and composed of hypertrophic chondrocytes (see inset, G'). (H) Labeled cells are mixed with unlabeled cells in Meckel's cartilage, but are not observed in the anterior and coronoid processes of the mandible. The perichondrium surrounding the process is composed almost solely of labeled cells. (I) Endogenous *dHAND* expression is not observed in the mandibular processes or in Meckel's cartilage, though expression is observed in the sympathetic ganglion (yellow arrow). Due to the angle of section, this structure is not observed in the *dHAND-Cre;R26R* or *dHAND-lacZ* embryo sections, though scattered labeled cells are present in the sympathetic ganglion of *dHAND-Cre;R26R* embryos (see Fig. 4). mo, molar.

dHAND expression. At E13.5, tooth development is at the bud stage, which is characterized by proliferative areas (composed of neural crest-derived mesenchyme) surround-

ing the dental bud that ultimately forms the enamel organ. As shown in Fig. 5, these proliferative areas were composed solely of labeled cells around both mandibular incisor (Fig.



5A) and molar (Fig. 5D) buds of *dHAND-Cre;R26R* embryos. Labeled cells were not observed around the maxillary incisor and molar buds (Fig. 5D and data not shown). β -gal-positive cells in E13.5 *dHAND-lacZ* embryos were also observed in the neural crest-derived mesenchyme surrounding the enamel organs of mandibular incisors (Fig. 5B), but were absent around the mandibular molars (Fig. 5E). Likewise, endogenous *dHAND* expression was only observed in the mandibular incisor mesenchyme surrounding the enamel organs (Fig. 5C). Expression was not observed in Meckel's cartilage (Fig. 5C) or around the mandibular molars (Fig. 5F). These findings thus indicate that the staining in the incisors of E13.5 *dHAND-Cre;R26R* embryos represents actual expression, while the staining around the molar buds represents fated expression.

By E15.5, odontogenesis has reached the cap stage, at which time the nonlabeled epithelial component of the developing tooth has begun to take the shape of the future tooth. Labeled cells comprising the dental papilla of both the mandibular molars and incisors were observed beneath the unlabeled epithelial cap in *dHAND-Cre;R26R* embryos (Fig. 5G and J). The relative number of unlabeled cells seemed to be higher in the molar papilla (arrow in Fig. 5G) than in the incisor papilla. The surrounding mandibular bone, symphysis of Meckel's cartilage, and mesenchyme were also labeled. β -gal-positive cells were also observed in the dental papilla of the incisors of *dHAND-lacZ* embryos, though were mixed with unlabeled cells in the symphysis of Meckel's cartilage (Fig. 5H). Labeled cells were again absent in the molar papilla (arrow in Fig. 5K). Endogenous

dHAND expression was only observed in the incisor dental papilla and surrounding mesenchyme (Fig. 5I). As observed at E13.5, expression was absent in the dental papilla of lower molars (Fig. 5L) and Meckel's cartilage (Fig. 5I), again suggesting differences in actual vs fated expression in the incisors and molars of *dHAND-Cre;R26R* embryos.

At E18.5, the distribution of *dHAND* progeny cells in the teeth of *dHAND-Cre;R26R* embryos was essentially unchanged. Dentin matrix, encompassing odontoblastic processes within dentinal tubules, showed strong staining in both developing mandibular incisors (Fig. 5M) and molars (Fig. 5P). Unlabeled cells were obvious in the dental papilla, likely reflecting the observed neural crest and nonneural crest contribution to the dental pulp (Chai et al., 2000). However, this mixing was once again more prominent in the molar pulp. The mandibular bone and surrounding mesenchyme were also composed of labeled cells. The entire enamel organ, including inner and outer enamel epithelial cells and the residual dental lamina, was unlabeled. Labeled cells were not observed in any part of the developing maxillary incisors (data not shown), though a few scattered cells were observed in the maxilla (data not shown).

In E18.5 *dHAND-lacZ* embryos, labeled cells were observed in the bone and mesenchyme surrounding the mandibular incisor dental papilla, with labeled cells in the symphysis of Meckel's cartilage mixed with unlabeled cells (Fig. 5N). While β -gal-positive cells were present in the mandibular bone surrounding the lower molars, labeled cells were not present in the molar dental papilla (Fig. 5Q). This pattern closely reflected the endogenous *dHAND* ex-

Fig. 4. Distribution of *dHAND* progeny cells in the middle ear and throat during development of *dHAND-Cre; R26R* embryos. Transverse (A–C; E13.5) and frontal (D–R; E18.5) sections through the middle ear and throat of *dHAND-Cre;R26R* (A, D, G, J, M, P), *dHAND-lacZ* (B, E, H, K, N, Q), and wild type (C, F, I, L, O, R) embryos. Treatment of sections was performed as described in Fig. 3. (A–C) Sections through the middle ear of E13.5 embryos. (A) In *dHAND-Cre;R26R* embryos, stained cells are observed in the mesenchyme surrounding the epithelium of the external auditory meatus (black arrow), in Meckel's cartilage (mc), and in the future manubrium of the malleus (m). Scattered labeled cells are also observed in the sympathetic ganglion (sg; see inset, A'). Labeled cells are not observed in the stapes (s), though are present in more proximal sections (see text). (B) Labeled cells in *dHAND-lacZ* embryos are also observed in the mesenchyme of the middle ear region, the manubrium of the malleus, and Meckel's cartilage, though they are mixed with unlabeled cells in Meckel's cartilage. No staining is observed in the stapes or sympathetic ganglion (see inset, B'). (C) Very little endogenous *dHAND* expression is observed in the middle ear. The exceptions are the sympathetic ganglion and the mandibular branch of the trigeminal ganglion (*). (D–R) Frontal sections through the heads of E18.5 embryos. (D) The tympanic (ty) and gonial (g) bones and Meckel's cartilage are composed almost solely of labeled cells in *dHAND-Cre;R26R* embryos. The surrounding mesenchyme in this region of the head is also composed of labeled cells. (E) Labeled cells in *dHAND-lacZ* embryos are observed in the tympanic ring and gonial bone, though a small portion of unlabeled cells are also present. More significant mixing of labeled and unlabeled cells is observed in Meckel's cartilage. (F) Little endogenous expression is observed in the tympanic and gonial bones and Meckel's cartilage, though faint expression is found along some of the head vasculature (arrow). (G, H) In sections through the malleus, labeled cells in *dHAND-Cre;R26R* and *dHAND-lacZ* embryos are confined to the manubrium (mb) of the malleus and surrounding mesenchyme. (I) *dHAND* expression is not observed in any part of the malleus or incus (i) in wild type embryos. (J, K) β -gal-stained cells in *dHAND-Cre;R26R* and *dHAND-lacZ* embryos are not present in the incus or stapes (s). Labeled cells in the manubrium of *dHAND-lacZ* embryos are mixed with unstained cells. (L) *dHAND* expression is not observed in the incus, stapes, or manubrium of the malleus. (M) In sections through the distal portion of the styloid cartilage (st), labeled cells in *dHAND-Cre;R26R* embryos compose most of the styloid and are scattered in the sympathetic ganglion (see inset, M'). (N) In *dHAND-lacZ* embryos, labeled cells are present in the perichondrium of the styloid cartilage, but are mixed in the cartilage itself and are absent in the sympathetic ganglion (see inset, N'). (O) *dHAND* expression is observed in the sympathetic ganglion in the incus/stapes region, with weaker expression in the glossopharyngeal ganglion. (P) In the throat, β -gal-stained cells in *dHAND-Cre;R26R* embryos are observed in the lesser horn (lh) and body (h) of the hyoid. However, labeled cells are mixed with unlabeled cells in the body, possibly reflecting changes in staining in cartilage undergoing endochondral ossification, as few hypertrophic chondrocytes are stained. Labeled cells are also observed in the mesenchyme of the submandibular and sublingual glands (black arrows). Out of the photographic frame, adipose tissue also contains scattered labeled cells (inset, P'). (Q) As with other cartilages, labeled cells in *dHAND-lacZ* embryos compose the perichondrium of the lesser horns of hyoid but are mixed with a majority of unlabeled cells in the cartilage itself. Very few labeled cells are observed in the body of the hyoid. Labeled cells are observed in the mesenchyme of the submandibular and sublingual glands (arrows), but are absent in adipose tissue observed out of the photographic frame (inset, Q'). (R) *dHAND* expression is not observed in or around the hyoid cartilages or bones; however, adipose tissue at the base of the neck shows strong *dHAND* expression. Out of the plane of section, expression is also observed in the submandibular gland (inset, R'). oc, otic capsule.

pression pattern, with weak *dHAND* signal observed in the mandibular incisor dental papilla, distal mandibular bone, and surrounding mesenchyme (Fig. 5O). No signal was detected in the mandibular molar or surrounding mesenchyme (Fig. 5R) or in Meckel's cartilage (Fig. 5O).

Discussion

Craniofacial development is regulated by multiple hierarchical signaling cascades that provide patterning and differentiation information to cephalic neural crest cells and their derivatives (Wilkie and Morriss-Kay, 2001). The transcription factor *dHAND* is likely involved in one or more of these cascades, based on previous studies of endogenous and transgenic expression patterns. We have shown here using fate analysis of *dHAND* progeny cells that *dHAND* likely plays crucial roles in development of neural crest-derived structures of the face, though based on our fate analysis and comparison with *dHAND-lacZ* expression and endogenous *dHAND* expression, the regulation of its expression is quite complex.

dHAND-Cre expression as a marker of current and past *dHAND* expression

One advantage of using *Cre* expression to examine gene expression patterns is the indelible nature of the staining, allowing observation of cell fate (Chai et al., 2000). These experiments also require cautious interpretation of resulting expression patterns, as transgenic expression constructs can lack elements necessary for complete gene expression or silencing. Indeed, analysis of the *Mrf4/Myf5* locus reveals pharyngeal arch enhancer elements 88 kb upstream from the transcription start site (Carvajal et al., 2001). In this study, we have shown that labeled cells in *dHAND-Cre;R26R* embryos are observed in almost all cranial structures in which endogenous *dHAND* expression is observed (this study and Howard et al., 2000; Srivastava et al., 1995, 1997; Thomas et al., 1998). This includes the sympathetic ganglion, where *dHAND-lacZ* expression was not detected (this study, and Charité et al., 2001). While expression of the *dHAND-lacZ* transgene in the -11.0 -kb line could be affected by transgene insertion effects, it is more likely that detection of *dHAND-Cre* expression is aided by the relative strength of the *ROSA26* promoter. The *ROSA26* gene is highly expressed in almost all embryonic and adult tissues (Soriano, 1997, 1999) and may therefore compensate for weak or partial enhancers, thus allowing weak *Cre* expression to be detected. This may be particularly true of the sympathetic ganglion expression, considering the scattered nature of staining in *dHAND-Cre;R26R* embryos vs the robust endogenous expression at E13.5. Using *dHAND-lacZ* deletion constructs, McFadden et al. (2000) demonstrated that, while the entire cardiac enhancer for *dHAND* was not

required for expression of *lacZ* in the heart, partial enhancers resulted in an overall attenuation of cardiac expression.

Labeled cells in *dHAND-Cre;R26R* embryos are observed in several locations that likely reflect ectopic expression or activation of enhancers within the *dHAND* upstream region, including the DRG and limb bud. Aberrant induction of transgene expression by local enhancers surrounding the integration site is well documented. In our previous *dHAND* promoter study, limb bud expression was occasionally observed in F0 transgenic embryos carrying different deletion constructs of the *dHAND* promoter (Charité et al., 2001). However, embryos generated with the 7.4-kb fragment consistently showed the same abnormal limb bud pattern as we have observed in our *dHAND-Cre* embryos (J.C. and E.N.O. unpublished data). It is possible that the limb bud represents a permissive environment for ectopic transgene expression, or that an enhancer within the -7.4 -kb upstream region acts in a promiscuous manner in the absence of the full *dHAND* upstream region.

Potential roles of *dHAND* during facial development

By comparing β -gal staining in *dHAND-Cre;R26R* and *dHAND-lacZ* embryos, we can begin to integrate the temporal expression of endogenous *dHAND* expression with overall *dHAND* progeny cell fate. In this study, we found labeled cells throughout the first pharyngeal arch of E10.5 *dHAND-Cre;R26R* and *dHAND-lacZ* embryos, with staining in E18.5 *dHAND-Cre;R26R* and *dHAND-lacZ* embryos observed in numerous neural crest-derived structures, including the mandible and Meckel's cartilage. It is slightly surprising that β -gal staining in E18.5 *dHAND-lacZ* transgenic mice, while weaker, is similar to that observed in E18.5 *dHAND-Cre;R26R* embryos. This likely does not reflect aberrant activity of the *dHAND* pharyngeal arch enhancer, as *dHAND*-specific expression of the *dHAND-lacZ* transgene along arch arteries three and four and their derivatives is correctly downregulated by E13.5 (unpublished observations). Rather, these current findings suggest that *dHAND* likely has multiple roles in facial development. The first probably occurs during patterning of neural crest cells and their derivatives that will eventually form the facial skeleton. It is at this time (E9.5–E10.5) that β -gal staining was first observed in the arches of both *dHAND-Cre;R26R* and *dHAND-lacZ* embryos, coinciding with the endogenous expression pattern of *dHAND*. Further, while *dHAND*^{-/-} embryos die by E10.5, disruption of mandibular development is already apparent (Thomas et al., 1998). Thus, it appears that *dHAND* function is crucial for the early development of postmigratory neural crest cells.

The second role appears to be later in jaw development, during which the *dHAND-lacZ* transgene is expressed in structures undergoing osteogenesis and chondrogenesis. In chondrogenic structures, labeled cells in E18.5 *dHAND-Cre;R26R* embryos are observed throughout the cartilage, whereas labeled cells in E18.5 *dHAND-lacZ* embryos are

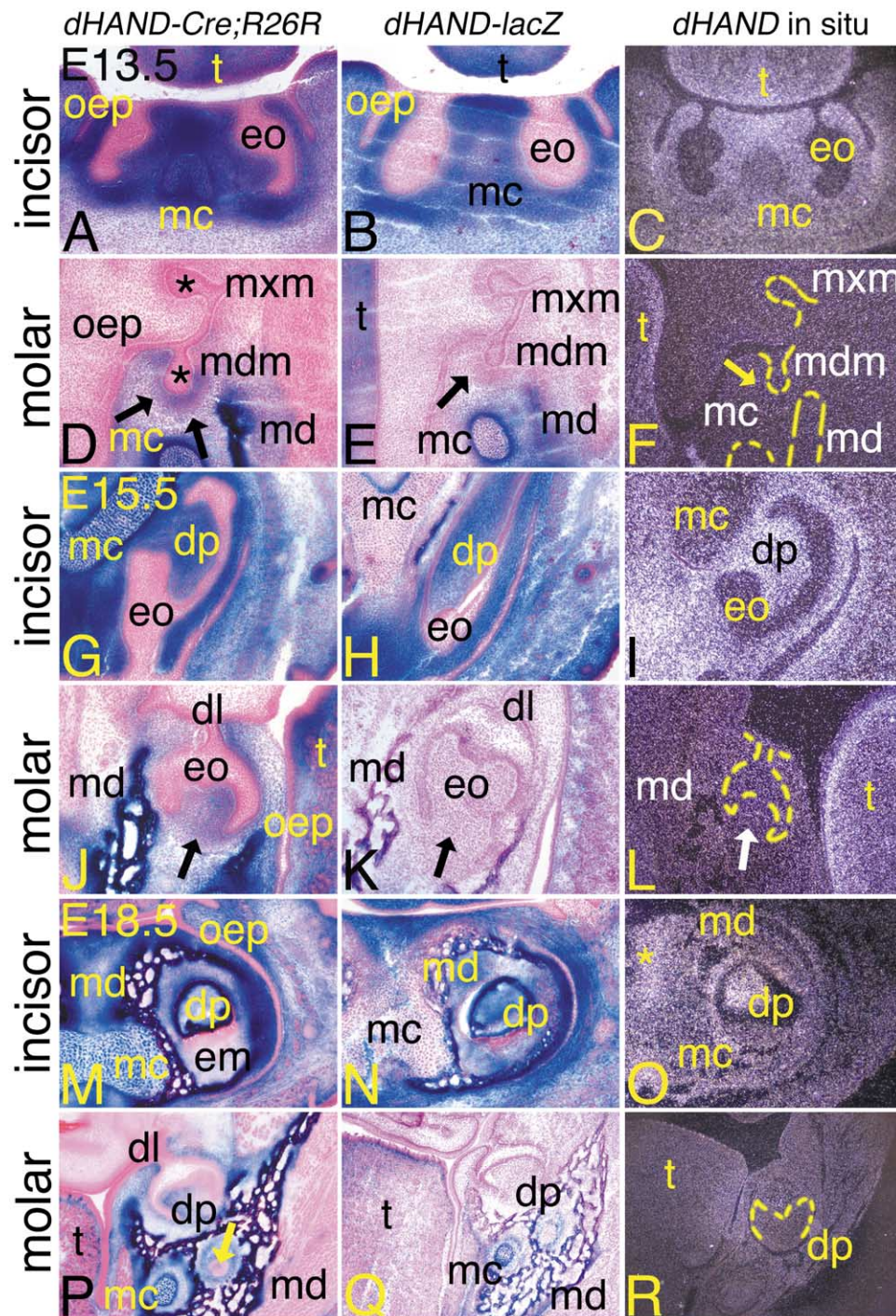


Fig. 5. Contribution of *dHAND* progeny cells during odontogenesis in *dHAND-Cre;R26R* embryos. Transverse (A–L) and frontal (M–R) sections through the heads of *dHAND-Cre;R26R* (A, D, G, J, M, P), *dHAND-lacZ* (B, E, H, K, N, Q), and wild type (C, F, I, L, O, R) embryos. Treatment of sections was performed as described in Fig. 3. (A–C) Transverse sections through the incisors of E13.5 embryos. (A, B) Labeled cells in *dHAND-Cre;R26R* and *dHAND-lacZ* embryos are apparent throughout the developing lower jaw, including in Meckel's cartilage (mc) and mesenchyme destined to become dentin and pulp of the lower incisors. Labeled cells are not observed in the oral epithelium (oep) or developing enamel organ (eo). (C) *dHAND* expression in wild type embryos is observed in the tongue (t) and mesenchyme surrounding the incisor enamel organs. Less expression is observed in Meckel's cartilage. (D–F) Transverse sections through the molar of E13.5 embryos, showing developing dentition of the molar at the late bud stage. (D) Labeled cells in *dHAND-Cre;R26R* embryos are observed in the mesenchyme surrounding the bud of the developing mandibular molar (mdm; arrows) and mandibular bone (md). Oral epithelium and developing enamel organs (*) of both maxillary (mxm) and mandibular molars are unlabeled. (E) Labeled cells in *dHAND-lacZ* embryos are not observed surrounding the mandibular molar buds (arrow). (F) Endogenous *dHAND* expression is also not observed surrounding the mandibular molar buds (arrow). (G–I) Transverse sections through the incisors of E15.5 embryos. The incisors have progressed to a cap stage, approaching the "bell" configuration. (G) The nonlabeled enamel organ in *dHAND-Cre;R26R* embryos partially encases the labeled dental papilla (dp) and appears irregular in shape due to the transverse angle of the section. The symphysis of Meckel's cartilage is almost completely composed of labeled cells. (H) Labeled cells

mainly confined to the perichondrium surrounding the cartilages. This suggests a limited role for dHAND in late chondrogenesis, such as possibly maintaining the proliferative environment surrounding developing cartilages in the jaw and throat. Past and active expression of the *dHAND* transgenes appears more equal in osteogenic structures in E18.5 embryos. Numerous genes and signaling pathways are involved in osteogenesis (Karsenty and Wagner, 2002); based on our current findings, it will be interesting to examine *dHAND* expression in mice lacking these genes to determine potential roles for dHAND in signaling pathways crucial for osteogenesis.

Positively defining the roles of dHAND, particularly in E18.5 embryos, is difficult due to the lack of correlation between endogenous *dHAND* expression and expression of a *dHAND-lacZ* transgene. Promoter-lacZ transgenic constructs or lacZ gene cassettes used to inactivate genes are routinely used to define gene expression patterns. However, we have shown here that β -gal staining is present even when endogenous gene expression is below the level of detection. This could reflect the relative stability of the *lacZ* message compared with that of the *dHAND* message. Further, while the half-life of dHAND protein is not known, in vitro experiments have shown that nuclear-localized β -galactosidase enzyme maintains 100% activity for at least 10 h (Tsuneoka and Mekada, 1992) and has a half-life of at least 20–30 h in cultured cells (Bachmair et al., 1986; Gonda et al., 1989). Because of this stability, it is quite likely that a low level of gene expression would result in a much larger pool of β -gal enzyme, thus making detection much easier compared with detection of mRNAs by in situ hybridization analysis. More study of this point needs to be conducted to fully understand the basis of these observations, and suggests that some caution should be taken when using β -gal staining to define temporal patterns of gene expression.

dHAND expression within subsets of cephalic neural crest cells

We have shown in this study that dHAND progeny cells compose most of the bone, cartilage, and connective tissue

of the lower jaw, suggesting a significant role for dHAND in cephalic neural crest cell development. However, while neural crest cells contribute to the cartilaginous precursors of malleus, incus, and stapes of the middle ear (Le Douarin, 1982; Noden, 1983, 1988), labeled cells in *dHAND-Cre; R26R* embryos are only observed in the manubrium of the malleus. This could indicate that one or more additional enhancers are required in addition to the arch enhancer in our construct to fully recapitulate the full *dHAND* expression domain within the arches. It is also possible that, while early *dHAND* expression appears quite broad, dHAND action within the neural crest derived ectomesenchyme is highly compartmentalized. Fate map analysis of chick neural crest cells illustrates that the malleus is derived from crest cells of different midbrain/hindbrain origins (Kontges and Lumsden, 1996). This presumably reflects both the origin and final spatial distribution of these cells within the arches, as recent studies suggest that neural crest cells are patterned by both signals in the neural tube and environmental signals encountered during migration into the arches (Trainor and Krumlauf, 2000; Trainor et al., 2002). It is possible that our results reflect a similar mixed contribution in mouse embryos, with dHAND (or at least individual dHAND enhancers) participating in the development of neural crest cells from specific midbrain/hindbrain origins or those residing in specific arch subregions.

dHAND expression during odontogenesis

Another aspect of facial development that was uncovered in our fate analysis was the difference in *dHAND* expression between incisors and molars. During early development, dHAND-progeny cells in *dHAND-Cre; R26R* embryos are observed in both mandibular incisor and molar mesenchyme and later, dental papilla, though actual gene expression is only observed in cells in the incisor region. As mentioned above, evidence of past expression (such as β -gal staining in *dHAND-Cre; R26R* embryos in the absence of current gene expression) in developing structures does not necessarily imply a direct function in structure formation. Further, neither incisor nor molar defects are observed in *ET_A^{-/-}* em-

dHAND-lacZ embryos also compose most of the dental pulp of the incisor, in contrast to the mixing of labeled and unlabeled cells in the symphysis of Meckel's cartilage. (I) *dHAND* expression is observed in the dental pulp and surrounding mesenchyme. Expression is much weaker in Meckel's cartilage symphysis. (J–L) Transverse sections through the molar of E15.5 embryos. (J) In the mandibular molar, also in the cap stage, labeled cells in *dHAND-Cre; R26R* embryos contribute to the dental papilla (arrow), though this area also contains unlabeled cells. (K) The dental papilla of mandibular molars from *dHAND-lacZ* embryos is composed solely of unlabeled cells (arrow). (L) *dHAND* expression is not observed in the papilla of the mandibular molars (white arrow), in contrast to the strong expression in the lamina propria of the tongue. (M–O) Frontal sections through the incisors of E18.5 embryos. (M) Labeled cells in *dHAND-Cre; R26R* embryos appear in the dental papilla of the mandibular incisors, mandibular bone (md), Meckel's cartilage, and surrounding mesenchyme. Oral epithelium remains unlabeled, as does the developed enamel matrix (em). (N) A similar pattern of staining is observed in the incisors of *dHAND-lacZ* embryos, though few labeled cells are observed in Meckel's cartilage. (O) *dHAND* expression is observed in the incisor dental pulp, surrounding bone, and mesenchyme. Very little expression is observed in Meckel's cartilage. (P–R) Frontal sections through the molars of E18.5 embryos. (P) Labeled cells in *dHAND-Cre; R26R* embryos appear in the dental papilla, although extensive mixing with unlabeled cells has occurred. Labeled cells are also observed in the mandibular bone, Meckel's cartilage, and surrounding connective tissue. The residual dental lamina (dl) can be seen connecting the unlabeled oral epithelium and enamel organ. Labeled cells are not observed in mandibular nerves (arrow). (Q) Labeled cells in *dHAND-lacZ* embryos are confined to the mandibular bone and Meckel's cartilage. Labeled cells are not observed in the dental papilla of the molar. (R) A low level of *dHAND* expression is observed in the mandibular bone surrounding the molar of wild type embryos, but expression is not observed in the molar dental pulp.

bryos, in which expression of both *dHAND* and *eHAND* is disrupted (Clouthier et al., 2000), suggesting that loss of these two transcription factors alone does not disrupt odontogenesis. However, as *dHAND* progeny cells are observed in both neural crest-derived incisor and molar dental papilla, it is tempting to speculate that *dHAND* provides early patterning information to crest cells that later populate the dental papilla of the mandible and that it has redundant functions with other mandibular arch genes. Studies of multigene knockout embryos will be required to better address this question.

In later development, *dHAND* expression remains in the mesenchyme and dental papilla of the incisors, but not in the molars. We cannot exclude the possibility that lack of molar expression is due to absence of one or more as yet unidentified arch enhancers required to fully recapitulate the endogenous *dHAND* expression pattern. However, distinct signaling pathways within the mandible are known to pattern the dentition within the maxilla and mandible (Ferguson et al., 2000; Thomas et al., 2000). It is possible that *dHAND* represents an “incisor-specific” gene, possibly playing roles in both late tooth development and positioning of the incisors within the mandible. Analysis of *dHAND* expression in mouse mutants with defects in incisor and/or molar development should help clarify *dHAND*’s role in odontogenesis.

dHAND-Cre mice as a tool to investigate developmental processes

Our present results demonstrate the power and utility of using Cre-based fate mapping studies to understand possible developmental functions of specific genes. More specifically, *dHAND-Cre* transgenic mice provide the opportunity to follow the fate of *dHAND* lineage neural crest and nonneural crest cells in different mouse mutant lines. Further, these mice should be useful in concert with conditional knockout mouse lines in dissecting the basis of multiple developmental and disease processes.

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